

The HLA–DRB1 Shared Epitope Alleles Are Primarily a Risk Factor for Anti–Cyclic Citrullinated Peptide Antibodies and Are Not an Independent Risk Factor for Development of Rheumatoid Arthritis

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Objective. The shared epitope (SE)–containing HLA–DRB1 alleles represent the most significant genetic risk factor for rheumatoid arthritis (RA). Recent studies indicate that the SE alleles are associated with only RA that is characterized by the presence of anti-cyclic citrullinated peptide (anti-CCP) antibodies, and not with anti-CCP–negative disease. In this study we investigated whether the SE alleles contribute to the development of anti-CCP–positive RA, or whether they are associated solely with the presence of anti-CCP antibodies. We therefore determined the influence of the SE alleles and anti-CCP antibodies on the progression from recent-onset undifferentiated arthritis (UA) to RA.

Methods. Patients with recent-onset UA at the 2-week visit (n = 570) were selected from the Leiden Early Arthritis Cohort. SE alleles, rheumatoid factor (RF) status, and anti-CCP antibody levels were determined. Progression to RA or other diagnoses was monitored.

Results. One hundred seventy-seven patients with UA developed RA during the 1-year followup, whereas the disease in 393 patients remained unclassified or was given other diagnoses. The SE alleles correlated with the presence of anti-CCP antibodies, but not with the presence of RF. Both in SE-positive and in SE-negative patients with UA, the presence of anti-CCP antibodies was significantly associated with the development of RA.

More intriguingly, however, no apparent contribution of the SE alleles to the progression to RA was found when analyses were stratified according to the presence of anti-CCP antibodies. In patients with anti-CCP–positive disease, the presence of SE alleles was associated with significantly higher levels of anti-CCP antibodies, suggesting that the SE alleles act as classic immune response genes.

Conclusion. The SE alleles do not independently contribute to the progression to RA from UA, but rather contribute to the development of anti-CCP antibodies.

The most important genetic risk factor for rheumatoid arthritis (RA) is the HLA–class II alleles. In particular, the HLA–DRB1 alleles encoding for the shared epitope (SE) confer a higher risk for the development of RA (1). The SE hypothesis postulates that the SE motif (a conserved amino acid sequence in the peptide binding pocket of the DRB1 molecule) is directly involved in the pathogenesis of RA by allowing the presentation of an arthritogenic peptide to T cells (2). Recently, it was observed by 2 different methods (linkage and association analyses) that the SE alleles are a risk factor for only RA that is characterized by the presence of anti-cyclic citrullinated peptide (anti-CCP) antibodies, and not for anti-CCP–negative RA (3).

Anti-CCP antibodies are highly specific for RA, can be detected years before the first clinical manifestation of RA (4), and are reported to be a good predictor for the development of RA (5). Because the contribution of the SE-containing HLA alleles to the pathogenesis of RA is not well understood, the novel information on the association of SE alleles with anti-CCP–positive disease (3) led us to evaluate the hypothesis that the SE alleles are mainly a risk factor for anti-CCP antibodies, rather than for (anti-CCP–positive) RA. To this end, we

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Table 1. Baseline characteristics of patients with undifferentiated arthritis at 2 weeks who did and those who did not develop RA during the first year of followup*

| | RA (n = 177) | Non-RA (n = 393) | <i>P</i> | OR (95% CI) |
|-----------------------------|-----------------|---------------------|----------|----------------|
| Age, mean \pm SD years | 56.3 \pm 15.3 | 48.6 \pm 16.9 | <0.001 | – |
| Sex, no. female/no. male | 121/56 | 208/185 | 0.001 | 1.9 (1.3–2.8) |
| SE positive, no. (%)† | 100 (63) | 158 (49) | 0.005 | 1.8 (1.2–2.6) |
| Anti-CCP positive, no. (%)‡ | 83 (51) | 38 (11) | <0.001 | 8.5 (5.2–13.7) |
| RF positive, no. (%) | 84 (47) | 56 (14) | <0.001 | 6.3 (4.1–9.7) |

* RA = rheumatoid arthritis; OR = odds ratio; 95% CI = 95% confidence interval; RF = rheumatoid factor.

† Data on shared epitope (SE) alleles were missing in 17 of the patients with undifferentiated arthritis (UA) progressing to RA and in 68 of the non-RA patients with UA.

‡ Anti-cyclic citrullinated peptide (anti-CCP) antibody data were missing in 15 of the patients with UA progressing to RA and in 49 of the non-RA patients with UA.

took advantage of a well-characterized inception cohort and studied the patients with an early arthritis that, at presentation, could not be classified according to the 1987 American College of Rheumatology (ACR; formerly, the American Rheumatism Association) criteria (6) (referred to as undifferentiated arthritis [UA]). Analysis of the clinical evolution in conjunction with the genetic and serologic risk factors in these patients who are prone to develop RA allows insight into the factors that are associated with progression to RA. Accordingly, this permits analysis of the contribution of the SE alleles to the development of RA after stratification for the influence of anti-CCP antibodies.

PATIENTS AND METHODS

Study population. For this study, patients were evaluated at the Leiden Early Arthritis Clinic (EAC), which was started in 1993 (for description, see ref. 7). Patients were referred to the EAC when arthritis was suspected, and were included in the cohort when arthritis was diagnosed at physical examination. At baseline, blood samples were obtained. More than 1,900 patients are currently included in the cohort.

Two weeks after inclusion, 313 patients received the diagnosis of RA according to the ACR 1987 criteria and 570 patients had an arthritis that could not be classified according to the ACR criteria and were therefore classified as having UA. After 1 year of followup, the disease status of all patients with UA was examined to determine whether they had developed RA according to the ACR criteria. It might be possible that some patients did not fulfill the ACR criteria for RA at 1 year but developed RA at a later time point. Inherent to the design of an inception cohort, the duration of followup will differ within the study population. However, at the time of this analysis, the majority of the patients (94%) had been followed up for more than 1 year (mean followup 8 years, SD 3 years), and only 9% of the patients who were not classified as having RA at 1 year developed RA later on in the disease course.

Laboratory investigations. Baseline laboratory parameters (determined using the blood samples that were obtained at inclusion) included IgM–rheumatoid factor (RF) by

enzyme-linked immunosorbent assay (ELISA) and anti-CCP-2 antibodies by ELISA (Immunoscan RA Mark 2; Euro-Diagnostica, Arnhem, The Netherlands). The cutoff level for anti-CCP antibody positivity was set at 25 arbitrary units, according to the manufacturer's instructions. The HLA–DRB1 subtyping was performed by polymerase chain reaction using specific primers and hybridization with sequence-specific oligonucleotides. The SE alleles were HLA–DRB1*0101, *0102, *0401, *0404, *0405, *0408, *0410, and *1001. For 438 of the 570 patients with UA, both data on SE alleles and data on anti-CCP antibodies were available.

Statistical analysis. Odds ratios (ORs) were calculated and proportions were compared by chi-square test. Differences in mean values between groups were analyzed using the Mann-Whitney test or *t*-test when appropriate. The question as to whether SE alleles and anti-CCP antibodies both independently contribute to progression to RA was investigated with a stratification procedure, as well as with logistic regression analysis. In this logistic regression analysis, the disease outcome was entered as the dependent variable and anti-CCP antibodies and SE alleles were possible explanatory variables. Using a backward selection procedure, the significant independent variables were selected. For all tests, *P* values less than 0.05 were considered significant.

RESULTS

Outcome in patients with UA. Of the 570 patients with UA at the 2-week visit, 177 developed RA during the first year of followup, 99 patients developed other rheumatic diseases (reactive arthritis, psoriatic arthritis, and systemic lupus erythematosus, among others), and 294 patients remained unclassified (having persistent UA). For further analysis, the patients with persistent UA and those with other diagnoses of rheumatic disease were described as the non-RA group. Characteristics of the patients who developed RA and of the patients in the non-RA group are given in Table 1. In univariate

Table 2. Comparison of patients with undifferentiated arthritis who, during 1 year of followup, did not and those who did develop RA, stratified for baseline anti-CCP antibodies and SE alleles*

| | Non-RA, no. (%) | RA, no. (%) | <i>P</i> | OR (95% CI) |
|-------------------------------|-----------------|-------------|----------|-----------------|
| Stratification for anti-CCP | | | | |
| Anti-CCP– | | | | |
| SE– | 142 (55) | 37 (53) | | |
| SE+ | 118 (45) | 33 (47) | 0.8 | 1.1 (0.6–1.9) |
| Anti-CCP+ | | | | |
| SE– | 8 (26) | 21 (27) | | |
| SE+ | 23 (74) | 56 (73) | 0.9 | 0.9 (0.3–2.6) |
| Stratification for SE alleles | | | | |
| SE– | | | | |
| Anti-CCP– | 142 (95) | 37 (64) | | |
| Anti-CCP+ | 8 (5) | 21 (36) | <0.001 | 10.1 (3.9–27.1) |
| SE+ | | | | |
| Anti-CCP– | 118 (84) | 33 (39) | | |
| Anti-CCP+ | 23 (6) | 56 (61) | <0.001 | 8.7 (4.5–17.0) |

* See Table 1 for definitions.

analysis, the presence of SE alleles, RF, and anti-CCP antibodies were all associated with significantly higher ORs for the likelihood of developing RA (ORs of 1.8, 6.3, and 8.5, respectively) (Table 1).

Association between SE alleles and presence of autoantibodies. To determine whether the SE alleles are correlated with RF positivity, with anti-CCP antibodies, or with both types of autoantibodies, the associations between the SE alleles and anti-CCP antibodies and between the SE alleles and RF were investigated in the 570 patients with UA. In univariate analysis, the SE alleles were associated both with RF and with anti-CCP antibodies (OR 1.7, 95% confidence interval [95% CI] 1.1–2.7, $P = 0.01$ and OR 3.1, 95% CI 2.1–5.3, $P < 0.001$, respectively).

Since anti-CCP positivity is correlated with RF positivity, the association between the SE alleles and anti-CCP antibodies was assessed in groups of patients stratified according to RF-positive and RF-negative disease. In patients with RF-negative disease, the presence of the SE alleles was associated with an increased likelihood of developing anti-CCP antibodies (OR 2.9, 95% CI 1.2–6.9, $P < 0.01$). Similarly, in patients with RF-positive disease, the presence of the SE alleles conferred an increased likelihood of having anti-CCP antibodies (OR 5.6, 95% CI 2.1–14.6, $P < 0.001$). These data indicate that the SE alleles are associated with the presence of anti-CCP antibodies independent of the RF status.

We next assessed whether the SE alleles are associated with the presence of RF independent of the presence or absence of anti-CCP antibodies. In both the anti-CCP-positive and anti-CCP-negative patient

groups, the SE alleles were not associated with the presence of RF ($P = 0.9$ and $P = 0.2$, respectively), indicating that after correction for the presence or absence of anti-CCP antibodies, the SE alleles do not confer a risk of RF positivity. Therefore, the SE alleles are primarily correlated with the presence of anti-CCP antibodies, but not with the presence of RF.

SE alleles and anti-CCP antibodies in progression from UA to RA. Subsequently, the influence of the SE alleles on the progression from UA to RA was examined. Univariate analysis assessing the association between patient characteristics and disease outcome revealed that the presence of the SE alleles and the presence of anti-CCP antibodies at baseline were both associated with the development of RA (see Table 1). However, because the presence of the SE alleles and the presence of anti-CCP antibodies are correlated, the individual effect of the SE alleles on the development of RA was determined after stratification for the presence or absence of anti-CCP antibodies. Both in the anti-CCP-positive and in the anti-CCP-negative patients with UA, the presence of the SE alleles was not associated with the development of RA (Table 2). These data are important because they indicate that the SE alleles are not correlated with progression to RA in patients with UA when corrections are made for the presence or absence of anti-CCP antibodies.

To assess the effect of anti-CCP antibodies independent of the SE alleles, the risk of developing RA was determined in SE-positive and SE-negative patients with UA in a separate analysis (Table 2). This analysis showed that both in the SE-positive and in the SE-negative patients with UA, the presence of anti-CCP

antibodies was significantly associated with the development of RA (OR 8.7 and OR 10.1, respectively).

In a logistic regression analysis with a backward selection procedure, with the disease outcome (RA versus non-RA) entered as the dependent variable and the SE alleles and anti-CCP antibodies as possible explanatory variables, the presence of anti-CCP antibodies was the only independent factor that was significantly associated with the development of RA, with an OR of 9.2 ($P < 0.001$). This result obtained from multivariate analysis was not substantially different from that obtained by univariate analysis in determining the influence of anti-CCP antibodies on the development of RA (OR 8.5) (see Table 1).

Thus, these data show that after stratification for the influence of the SE alleles, the presence of anti-CCP antibodies confers a high risk for the development of RA, whereas after stratification for the presence or absence of anti-CCP antibodies, the SE alleles are not associated with progression to RA. Taken together, these data indicate that the SE alleles primarily predispose to the presence of anti-CCP antibodies, and are not an independent risk factor for the development of RA.

Association between SE alleles and anti-CCP antibody level. In classic studies, performed in mice, on the genetic background associated with antibody production, it has been shown that major histocompatibility complex (MHC) alleles act as immune response genes that control the magnitude and specificity of antibody production in a dominant manner (8). In mice, the magnitude of the antibody response in the first generation offspring was comparable with the magnitude of response in the high-responding parent, denoting that in mice, homozygosity for MHC genes did not improve the level of antibody production compared with that in a heterozygous background (8).

Because the results of the present study revealed that the presence of the SE alleles is associated with positivity for anti-CCP antibodies, we wished to investigate whether the characteristics of the SE alleles resemble those of a classic immune response gene. We therefore analyzed whether the level of anti-CCP antibodies present in serum was correlated with the presence of the SE alleles. To this end, the correlation between the presence of the SE alleles and the level of anti-CCP antibodies was assessed in all anti-CCP-positive patients who, at the 1-year followup, had progressed to having RA. Of a total of 490 RA patients (313 with RA diagnosed at 2 weeks' followup and 177 patients whose condition progressed from UA to RA during the first

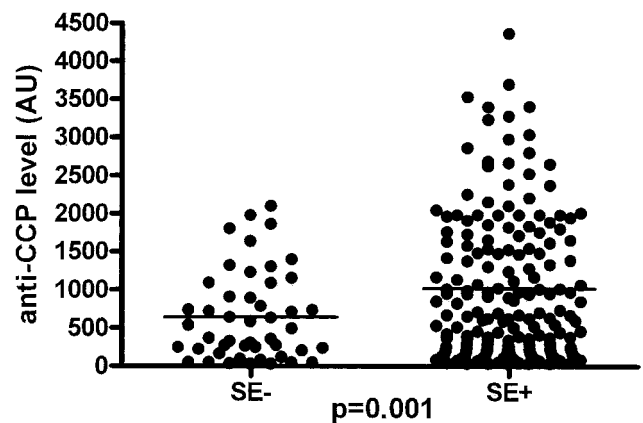


Figure 1. Levels of anti-cyclic citrullinated peptide (anti-CCP) antibodies (in arbitrary units [AU]) in anti-CCP-positive patients with rheumatoid arthritis (RA) without and those with shared epitope (SE) alleles. Bars indicate the median anti-CCP antibody level. The mean anti-CCP antibody levels in the anti-CCP-positive RA patients were 1,041 (SEM 134) for those carrying 2 SE alleles ($n = 46$), 1,029 (SEM 86) for those carrying 1 SE allele ($n = 123$), and 652 (SEM 86) for those carrying no SE alleles ($n = 46$). In the subgroup of anti-CCP-positive patients with undifferentiated arthritis that progressed to RA, the median anti-CCP antibody levels were 699 (interquartile range [IQR] 278–1,282) for those carrying 2 SE alleles ($n = 13$), 927 (IQR 251–1,970) for those carrying 1 SE allele ($n = 43$), and 358 (IQR 169–1,424) for those carrying no SE alleles ($n = 21$).

year of followup), 233 patients had anti-CCP antibodies, of whom 73% carried SE alleles.

The anti-CCP antibody levels in the anti-CCP-positive, SE-positive and anti-CCP-positive, SE-negative patients are shown in Figure 1. SE-positive patients had a significantly higher level of anti-CCP antibodies ($n = 169$, mean 1,032 arbitrary units, SEM 72) than did SE-negative patients ($n = 46$, mean 652 arbitrary units, SEM 86) ($P = 0.001$). Patients carrying 1 SE allele displayed a significantly higher level of anti-CCP antibodies ($n = 123$, mean 1,029 arbitrary units, SEM 86) compared with patients without SE alleles ($P = 0.002$). Patients with 2 SE alleles did not have a significantly higher anti-CCP level ($n = 46$, mean 1,041 arbitrary units, SEM 134) compared with patients carrying 1 SE allele ($P = 0.94$).

Thus, the current data show that in anti-CCP-positive patients, the presence of SE alleles is associated with higher levels of anti-CCP antibodies, and indicate that the presence of 1 or 2 SE alleles does not result in an apparent difference in anti-CCP antibody level. This observation is compatible with the notion that the SE alleles function as immune response genes in the development of anti-CCP antibodies.

DISCUSSION

We recently reported (3) that the SE alleles were only associated with anti-CCP-positive RA and not with anti-CCP-negative disease, indicating that the SE alleles are not associated with RA as such, but rather with a distinct phenotype of the disease. We now extend these findings by showing that the SE alleles are not an independent risk factor for the development of RA after correction for anti-CCP antibody status. The SE alleles were, however, associated with the presence of anti-CCP antibodies. Moreover, the presence/absence of SE alleles was correlated with the levels of anti-CCP antibodies, suggesting that the SE alleles act as classic immune response genes for the development of anti-CCP antibodies.

Although no formal conclusions on causality can be drawn from this association study, these findings suggest that anti-CCP antibodies mediate the association between SE alleles and RA. It would be of interest to replicate the findings of the present study by following the development of anti-CCP antibodies and RA in healthy asymptomatic persons with and without SE alleles. Nevertheless, the present findings constitute an important refinement of the long-known association between HLA and RA by indicating that the SE alleles are not primarily associated with RA, but rather with anti-CCP antibody positivity.

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